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# TYROSINE KINASE INHIBITOR TYRPHOSTIN AG490 INHIBITS OSTEOCLAST DIFFERENTIATION IN COLLAGENASE-INDUCED OSTEOARTHRITIS

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**The janus kinase (JAK)-signal transducer and activator of transcription (STAT) cascade plays a principal role in the signaling of a vast array of cytokines and growth factors which stimulates diverse cellular functions and immune responses. Osteoarthritis (OA) is the most common joint disease in the adult population. The present study was designed to evaluate the effects of tyrosine kinase inhibitor, tyrphostin AG490 in a mouse model of collagenase-induced osteoarthritis (CIOA). CIOA was provoked by two intraarticular (i.a.) injections of collagenase in mice and intraperitoneally (i.p.) treated with AG490 at a dose of 5 mg/kg at days 0, 5 and 10 and at a dose of 8 mg/kg at day 18. The administration of AG490 in CIOA mice inhibited osteoclast generation in bone and the loss of glycosaminoglycans and proteoglycans in cartilage. Tyrphostin decreased the levels of IFN-γ, IL1, IL-6 and IL-17 in the synovial fluid (SF) dependant on the time post AG490 administration. Limited numbers of CD11b positive Ly6G neutrophils in blood and SF along with a decrease of F4/80 positive cells in synovial fluid (SF) were observed in tyrphostin AG490-treated arthritic mice. AG490 inhibited M-CSF+RANKL-induced cytokine production by bone marrow (BM) cells and the differentiation of BM cells *in vitro*. Because of the findings presented, we argue that tyrphostin AG490 may hold promising therapeutic potential against important clinical conditions such as osteoarthritis (OA).**

Osteoarthritis is a chronic joint disorder that requires long-term treatment. Although OA is mainly considered as a degradative condition of the articular cartilage, there is an increasing body of evidence demonstrating the involvement of different branches of the immune system. The initial injury of cartilage triggers the activation of immune responses including T cell, B cell and macrophage infiltration into the joint tissues, cytokine and chemokine release by different kinds of cells present in the joint, resulting in further damage of the articular cartilage (1). While there is considerable success in the treatment of rheumatoid arthritis (RA) using anti-cytokine therapies in OA, however, these therapies do not show a pronounced effect. This needs the development of more novel approaches to treat OA aiming at therapies that act on multiple targets. The main characteristics of OA are cartilage degradation, synovial fibrosis, and formation of osteophytes as a response to excessive joint loading or impaired metabolic environment in OA joints. Frequently, osteophytes lead to deformation of the joint, compression of neighboring tissue and decrease of joint function (2).

The imbalances between pro-inflammatory and anti-inflammatory cytokine activities favor the induction of chronic inflammation and joint damage (3). Thus, agents capable of inhibiting the generation of pro-inflammatory mediators are considered as

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candidate therapies of OA. Bone erosion is based on the differentiation of mononuclear precursor cells to mature osteoclasts within the synovial membrane (4, 5). By using knock-out mice models the pivotal role of osteoclasts for bone destruction has been clearly proven (6, 7). This suggests that an intervention limiting the attack by inflammatory synovial tissue may be based on the inhibition of osteoclast differentiation and activity.

Tyrosine kinases play a central role in signal transduction through transfer of the terminal phosphate group of ATP to the tyrosine residues on protein substrates, resulting in activation of the substrate. Subsequently, signal transducers and activators of transcription (STATs) are activated and act as transcription factors (8, 9). Protein tyrosine kinase inhibitors (PTKIs) were first investigated as anti-cancer agents and then used in clinical trials (10). Now, they have been evaluated in various animal models of multiple sclerosis (11), intestinal inflammation (12), and septic shock (13). Recently, over 20 drugs that target kinases have been introduced in medical practice and many are currently in preclinical studies (14). Mitogen-activated protein kinases (MAPKs) and non-receptor tyrosine kinases such as spleen tyrosine kinase (Syk) and JAKs mediate the signaling and transcription of proinflammatory genes triggering the synthesis of many cytokines like interleukin (IL)-1, IL-2, IL-12 and interferons (IFNs) (15). The *in vitro* studies revealed several mechanisms of their actions, one of them being the inhibition of Janus kinase (JAK)2 pathway (16, 17) associated with the phosphorilation of STAT1 and STAT3 (18). Inhibition of tyrosine phosphorilation, thereby changing cell signaling, tyrosine kinase inhibitors can influence multiple inflammatory pathways. However, none of the kinase inhibitors is fully specific. For example, one of tyrosine kinase inhibitors, tyrphostin AG490 has been considered as a selective inhibitor of the JAK2 and JAK3/STAT pathways, while it does not inhibit other lymphocytic tyrosine kinases such as Lck, Lyn, Btk, Syk, Src, or Tyk2 (19). Additionally, it was found that AG490 blocked the activation of B cell precursors of acute lymphoblastic leukemia (ALL) patients through inhibition of JAK3/AP-1 and JAK3/MAPK phosphorilation (20) and MAPK pathway in human multiple myeloma cells (21).

Collagenase-induced osteoarthritis (CIOA) is an animal model of osteoarthritis that has been used to address questions of disease pathogenesis. The main pathological features of CIOA include synovitis with infiltration of inflammatory cells, cartilage degradation, bone erosion and osteophyte formation (22). Previously, we showed that tyrphostin AG490 has beneficial effects on a model of septic shock induced by zymosan. At a single dose of application (5 mg/kg) the substance increased the rate of survival and ameliorated the signs of multiple organ failure (23). In another investigation, we observed that tyrphostin AG490 diminished cartilage and bone destruction in CIOA model. This effect was receptor activator of nuclear factor κB ligand (RANKL)-dependant, since a decrease of RANKL expression in the joints of arthritic mice and on synovial fluid cells was observed. AG490 inhibited RANKL-induced osteoclast differentiation *in vitro* (24). The aim of the present study is to further define the mechanism of anti-osteoclastogenic effects of tyrphostin AG490 in a model of collagenase-induced osteoarthritis.

## MATERIALS AND METHODS

*Mice*

Male ICR mice (CD-2) background (8-10 week old, weight 20-22 g) were bred in the Animal Facility of the Institute of Microbiology), maintained on a 12:12 h light:dark cycle and fed standard diet and tap water *ad libitum*. All experiments were conducted in accordance with the Bulgarian National Guidelines for the Care and Use of Laboratory Animals (Decree No. 14/19.07.2000) and were approved by the Animal Care Committee at the Institute of Microbiology, Sofia.

Tyrphostin AG490 (2-Cyano-3-dihyroxyphenyl)-N-(benzyl)-2-propenamide (Sigma-Aldrich, St. Louis, USA) was dissolved in DMSO and further diluted in PBS (final DMSO concentration was <0.05%).

*Collagenase-induced osteoarthritis and treatment*

After one week of acclimation, mice (n=7 per group in 3 experiments) were anesthesized by i.p. injection of sodium pentobarbital at a dose of 50 mg/kg and then received two i.a. injections of collagenase from *Clostridium histolyticum* (each injection of 1 U/mice; Sigma-Aldrich, Diesenhofen, Germany) at day -2 and 0. Control animals were injected i.a. with 10 μl endotoxin-free phosphate-buffered saline (PBS; Lonza, Verviers, Belgium).

One group was treated with tyrphostin at a dose of 4 mg/kg at days 0, 5 and 10 and with 8 mg/kg at day 18. The course of CIOA can be divided into onset (between day 0 and day 5), developing inflammation with active processes around day 18, and established phase until day 30. Previously, it was established that the substance was effective at a single dose of 5 mg/kg in a model of multiple organ dysfunction syndrome (23). Thus, we aimed to target the initiation (day 0), onset (days 5 and 10) at a lower dose of 4 mg/kg for repeated treatment and we administered a two-fold higher dose at the most active point (day 18). Control animals were injected i.p. with DMSO/PBS solution instead of AG490 under this scheme. Histopathological changes and different cell populations in the synovial fluid were determined at day 30.

*Flowcytometry*

Blood was collected by retro-orbital puncture (n=10 per group in 2 experiments) in tubes containing 5 U/ml of heparin and mixed with an equal volume of PBS (pH 7.4). After gradient centrifugation on Histopaque 1083Ô (Sigma-Aldrich) at 1400×*g* for 40 min at room temperature, peripheral blood mononuclear cells (PBMCs) were carefully collected, washed with PBS and counted. For SF cell collection the skin overlying the ankle was excised. A 30-gauge needle was inserted through the synovial membrane, and the synovial cavity was washed by injecting and immediately aspirating 25 µl of heparinized PBS (5 U/ml) to obtain the synovial lavage and then centrifuged. SF cells and PBMCs were resuspended at 2 x $10^5$/ml in 2% FCS/PBS solution. The cells were incubated for 15 min at 4°C with appropriately diluted antibodies from Biolegend (San Diego, USA) against mouse Ly-6G (FITC-conjugated; clone 1A8), CD11b (PE-conjugated; clone M1/70) and F4/80 (FITC-conjugated, clone BM8) and IgG isotype controls (BD Bioscience). After washing with 2% FCS/PBS, the samples were analyzed with flow cytometer (BD LSR II) by using BD FACSDiva v6.1.2 Software (Becton Dickinson GmbH, San Jose, CA, USA).

*Histological analyses*

Hind paws were removed at day 30 of CIOA, fixed by incubation in 10% buffered formaldehyde for 48 h and decalcified by incubation in 10% EDTA, 0.2% paraformaldehyde (PFA) in PBS at 4°C, with the decalcifying solution changed twice weekly, for three weeks. The paws were embedded in paraffin, serial sections (8 µm) were cut, mounted on glass slides and stained with toluidine O or 0.1% safranin O/fast green. All histological assessments were performed in a blinded fashion. For TRAP staining, sections were incubated for one hour in 1 mg/ml naphthol AS-TR phosphate (N-(4-Chloro-2-methylphenyl)-3-(phosphonooxy)naphthalene-2-carboxamide), 60 nmol/l NN-dimethylformamide, 100 nmol/l sodium tartrate, and 1 mg/ml Fast red TR salt solution (all from Sigma-Aldrich, St. Louis, USA). The number of osteoclasts was determined at high magnification (× 100) and the results were expressed as the total number of osteoclasts per slide. Toluidine blue–stained sections were examined to measure the areas of total and negatively stained cartilage to show glycosaminoglycan (GAG) distribution and loss. The proportion of damaged cartilage was calculated by dividing the area of negatively stained cartilage by the area of total cartilage. Safranin-O proteoglycan (PG) loss was graded as follows: score 0, intact surface; score 1, minimal loss; score 2, moderate loss with surface fissures to midzone; score 3, marked loss with surface fissures to deep zone; score 4, complete disorganization.

*Determination of cytokines*

Synovial fluid was harvested by lavage of ankles with 25 µl of PBS/1 mM EDTA. Plasma was obtained after centrifugation of heparinised blood. The amounts of IFN-g (Abcam, Cambridge, UK), IL-1, IL-6 and IL-17 (Biolegend, UK) were quantified by ELISA kits according to manufacturer's instructions.

*Osteoclast differentiation*

Bone marrow-derived macrophages were isolated from the long bones of 6-week-old mice. Using a 27G needle/1 ml syringe filled with α-minimal essential medium (Sigma-Aldrich) the bone marrow from both ends of the bone was flushed. The suspension was gently aspirated to disrupt cell aggregates and was centrifuged at 1800xg for 5 min. Bone marrow precursors ($2x10^6$/ml) were cultivated in α-minimal essential medium supplemented with 10% FCS (Sigma-Aldrich), antibiotics (gentamicin 50 µg/mL, penicillin G 100 µg/ml, Sigma-Aldrich), with 30 ng macrophage colony stimulating factor (M-CSF) (GenScript) for 1 day, followed by 50 ng M-CSF for 3 days and then with 50 ng M-CSF + 50 ng sRANKL (Biolegend, UK) for a further 3 days. One group of cells was cultivated in the absence of AG490 and another group was cultivated in the presence of 50 mM AG490. The cells were fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 5 min, and then permeablilzed with 0.5% Triton X-100 in PBS for another 5 min. Subsequently, fixed cells were incubated with 0.01% naphthol AS-MX phosphate (Sigma) and 0.05% Fast Red Violet LB salt (Sigma) in the presence of 50 mM sodium tartrate and 90 mM sodium acetate (pH 5.0) for 5 min. The TRAP-positive cells containing more than four nuclei were considered osteoclasts.

*Statistical analyses*

Statistical analyses were performed using InStat 3.0

and GraphicPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The data were presented as the mean ± SD. Group mean values were compared by Student's unpaired *t*-test. Differences were considered significant when $P < 0.05$.

## RESULTS

*The effect of AG490 on osteoclast generation and cartilage loss in CIOA*

Fig. 1A presents histologic sections of hind paws from untreated and AG490–treated CIOA mice. Untreated mice showed numerous osteoclasts at the border between cartilage and bone, and also deep in the bone at the sites of erosion. Tyrphostin significantly inhibited osteoclast generation (Fig. 1B). Fig. 1C illustrates representative cartilage specimens with advanced GAG loss in the joints of CIOA mice. In contrast, mice treated with tyrphostin AG490 showed good preservation of cartilage glycan content (Fig. 1D). Safranin O staining also showed the extensive deep cartilage degradation in several locations retarded by AG490 (Fig. 1E). Mean score

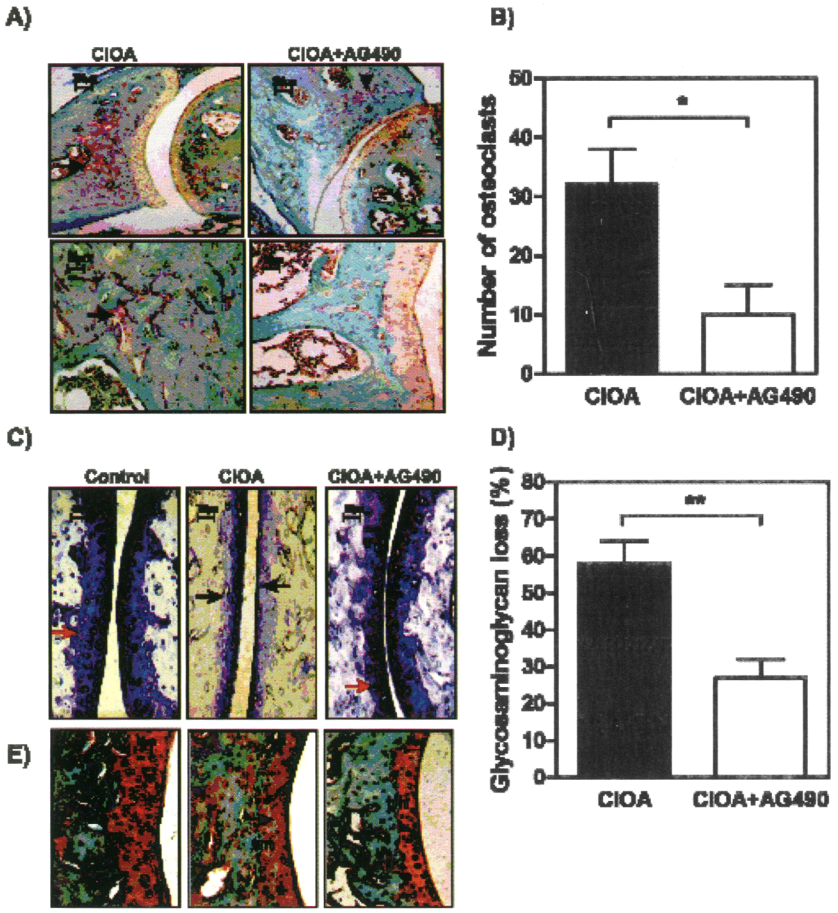



**Fig. 1.** *Tyrphostin AG490 inhibited osteoclast generation in the bone and decreased cartilage loss. Ten joint sections per mouse (n=7 per group).* ***A)*** *Representative TRAP (tartrate resistant acid phosphatase positive cells) stained joint sections. Arrows indicate TRAP positive cells in CIOA mice.* ***B)*** *Number of osteoclasts (multinucleated TRAP positive cells) in the bone. *P < 0.05, Student's unpaired t- test.* ***C)*** *Tyrphostin AG490 reduced GAG thinning (toluidine blue staining). Black arrows indicate destained articular cartilage; red arrows indicate maintained glycan content.* ***D)*** *Percentage of distained areas showing cartilage loss.* ***E)*** *Control cartilage had intact superficial, mid and deep zones that demonstrate intense, uniform staining with safranin O for PG. CIOA group showed increased matrix PG depletion and matrix erosion (arrow), loss of tidemark (tm) zone, and loss of viable chondrocytes (asterisk). **P < 0.01, Student's unpaired t-test. Scale bar = 50 mm or 200 mm.*

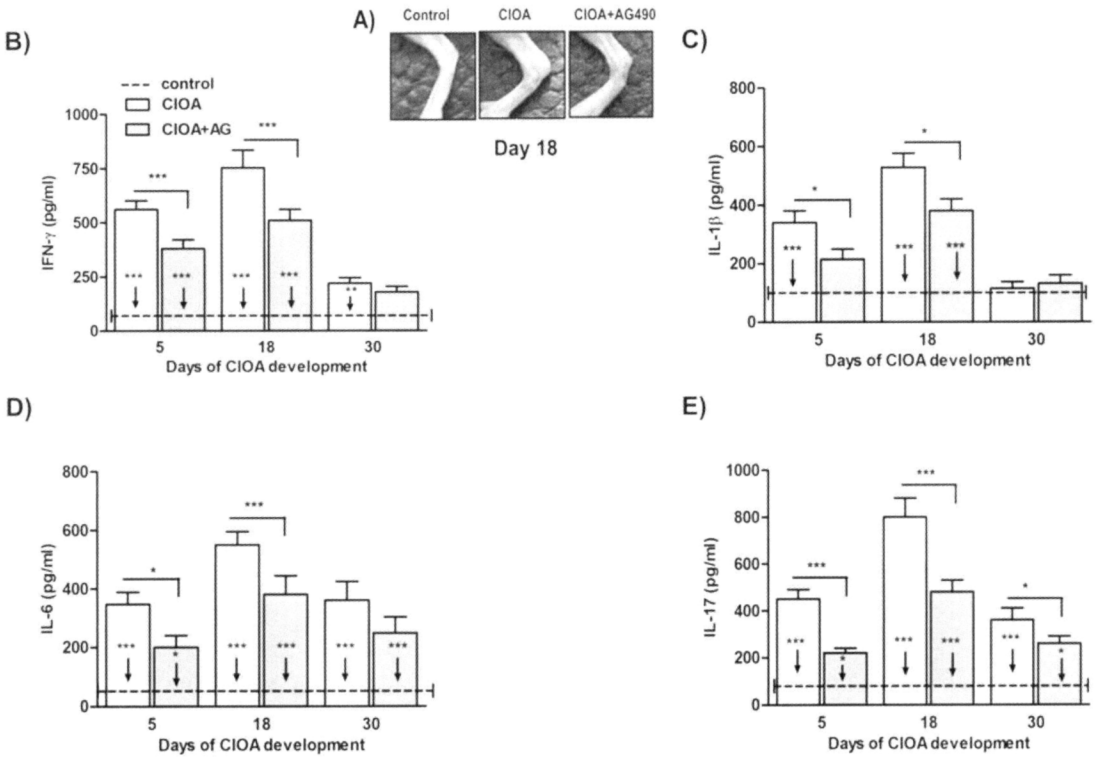



**Fig. 2.** *Cytokine levels in the synovial fluid at different time points of CIOA determined by ELISA. Tyrphostin AG490 was injected at a dose of 4 mg/kg at days 0, 5, 10 and at a dose of 8 mg/kg at day 18.* ***A)*** *AG490 inhibited joint inflammation at day 18 of CIOA. AG490 lowered IFN-γ* ***(B)****, IL-1β (C), IL-6* ***(D)*** *and IL-17* ***(E)*** *levels. Data are presented as means ± SD from 3 determinations (n = 7 per group). *P < 0.05; **P < 0.01; *** P < 0.001, Student's unpaired t-test, arrows indicate comparison to controls.*

for PG loss was 3.5±0.5 for the CIOA group and 2.0±0.4 for the AG490-treated group ($P < 0.001$)

*Administration of AG490 inhibits cytokine levels in SF of CIOA mice*

Cytokines have been shown to play a pivotal role in arthritis and in rodent models, and we therefore investigated the effect of AG490 on the production of selected cytokines in the synovial fluid. We aimed to see the changes at those points when active inflammation was observed (days 5 and 18) and when osteoarthritis was at the established phase (day 30). Fig. 2A shows the macroscopically inflamed joint of mice at day 18 of CIOA which was suppressed in AG490-treated mice. Tyrphostin AG490 increased IFN-γ levels on day 5 and 18 but did not change IFN-γ concentration on day 30 (Fig. 2B). Elevated concentrations of IL-1 in SF were found at days 5 and 18 in CIOA mice, significantly suppressed in the tyrphostin-treated group (Fig. 2C). In regard to synovial IL-6, AG490 abolished their levels at days 5 and 18 but did not cause significant changes at day 30 (Fig. 2D). Tyrphostin diminished the level of IL-17 in SF at all time points tested (Fig. 2E). In general, day 18 proved to be related with the highest cytokine levels in the SF.

*Tyrphostin AG490 reduces the number of CD11b positive Ly6G neutrophils and F4/80 macrophages*

At day 30 of CIOA we determined the number of CD11b+/Ly6G+ cells in the SF and the expression of these markers on PBMCs. Data showed that the elevation of double positive cells in CIOA mice was diminished in the group treated with AG490, in blood (15.47±1.20, 66.71±4.79 and 42.7±4.15 for control, CIOA and CIOA+AG490, respectively)

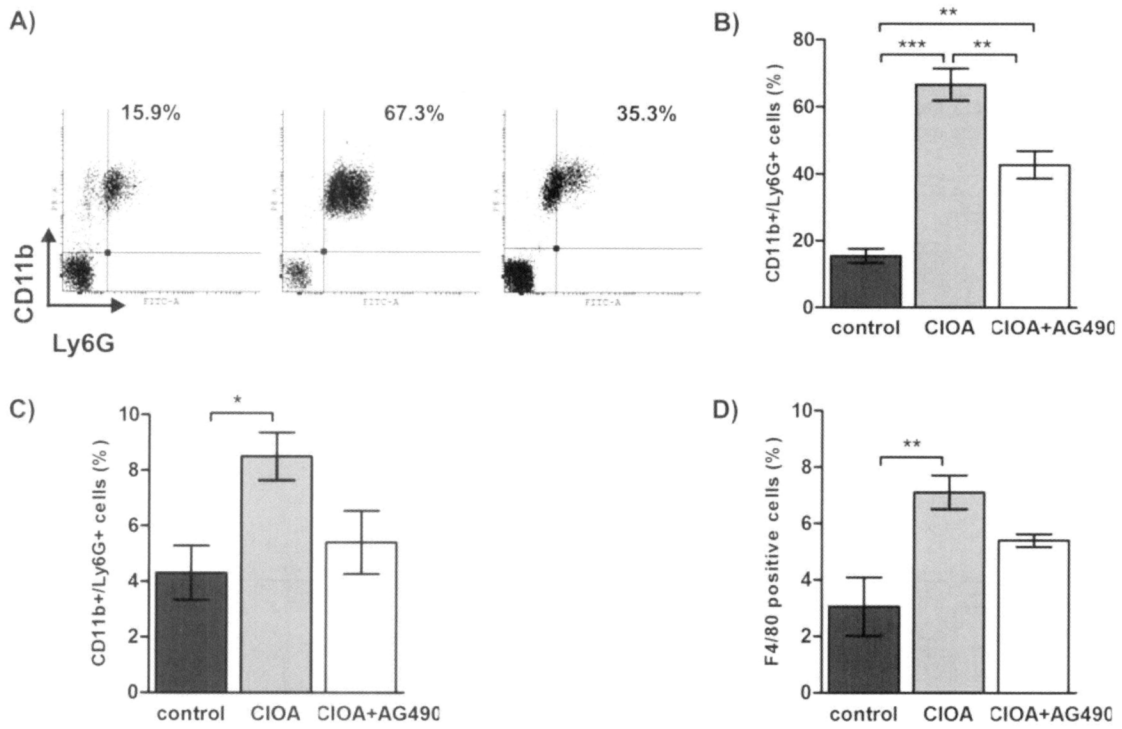



**Fig. 3.** *Effect of AG490 on the number of CD11b+Ly6G+ and F4/80 positive cells. Tyrphostin AG490 was injected as described in Fig. 2. Blood and SF were collected at day 30 of CIOA (n = 10 per group in 3 experiments).* ***A)*** *A representative dot-plot histogram and figure data* ***(B)*** *showing decreased frequencies of CD11b+Ly6G+ cells in blood after AG490 treatment. Flowcytometry analyses showed that tyrphostin suppressed the elevation of CD11b+Ly6G+ cells in the SF* ***(C)*** *and decreased the number of F4/80 cells in SF* ***(D)****. Values are means ± SD (n=10 per group in 3 experiments). *P < 0.05; **P < 0.01; *** P < 0.001, Student's unpaired t-test.*

(Fig. 3A, B). The same effect was observed in SF (4.30±0.55, 8.50±0.86 and 5.40±1.13) (Fig. 3C). An increased number of F4/80+ cells was observed in the SF of CIOA mice (7.11±0.60) compared with the control group (3.05±0.59), substantially inhibited by tyrphostin AG490 (5.40±0.23) (Fig. 3D).

*Tyrphostin AG490 alters cytokine production by BM cells and inhibited osteoclast diffrentiation*

Bone marrow cells were isolated from non-arthritic and CIOA mice at day 18 of arthritis and cultivated *in vitro* with M-CSF and RANKL in the presence or absence of AG490. The supernatants were collected for cytokine determination at each time point when media was changed. At day 1 (30 ng M-CSF presented during cultivation) IL-1 release was higher in the CIOA group compared to the control group, while AG490 reduced cytokine production. At day 4 (50 ng M-CSF presented during cultivation) tyrphostin inhibited IL-1 production in the CIOA group and had no effect on the control group. On day 7 (50 ng M-CSF + 50 ng RANKL presented during cultivation) a low concentration of IL-1 was determined with no differences between the groups (Fig. 4A-C). In regard to IL-6 the most profound production was observed at day 7. Tyrphostin AG490 significantly suppressed its release at all time points (Fig. 4D-F).

At day 7 the cells were collected and subjected to FACS analyses. We observed that F4/80 cells consisted of $F4/80^{low}$ and $F4/80^{high}$ populations. Results showed that the percentage of $F4/80^{low}$ population was not influenced by tyrphostin, while less $F4/80^{high}$ population were observed in the non-arthritic group (Fig. 5A). When cells were obtained from CIOA mice, the frequencies of $F4/80^{low}$ population increased approximately two-fold, and this elevation

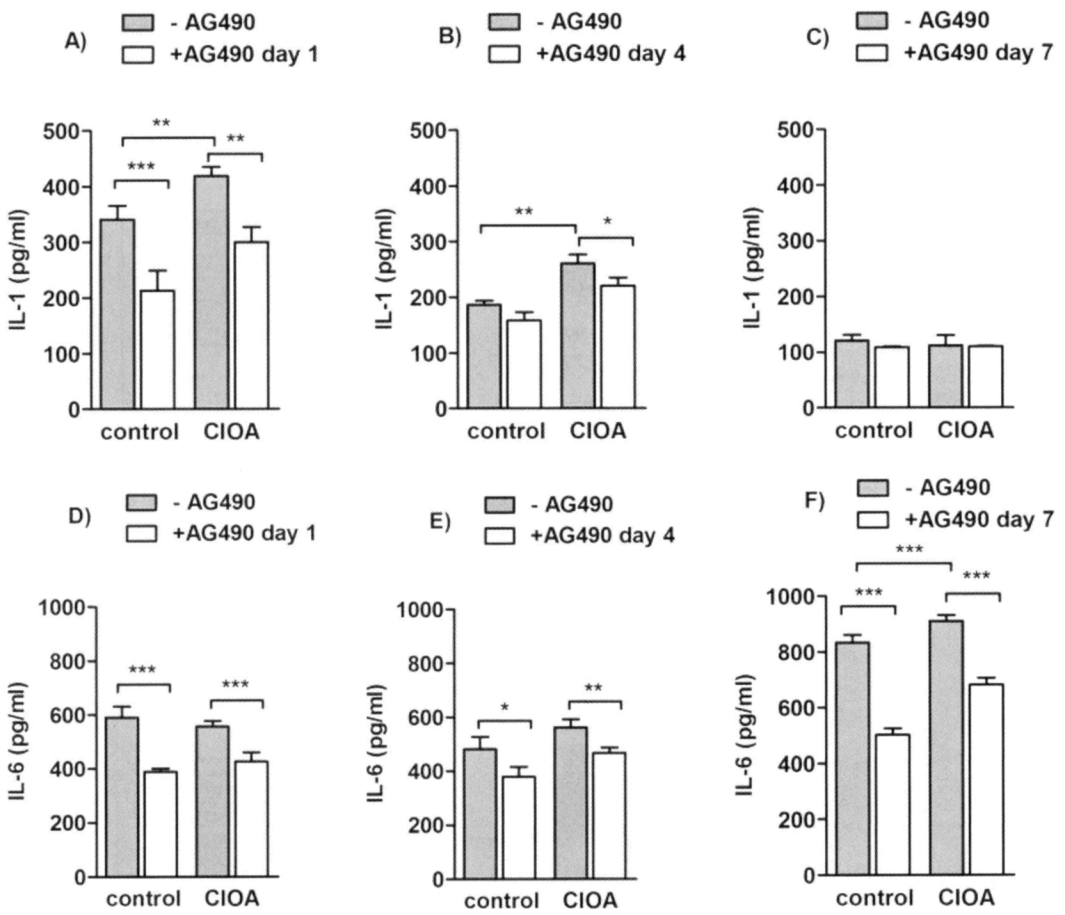



**Fig. 4.** *Effect of tyrphostin AG490 on IL-1 and IL-6 production. BM cells obtained from control and from mice with CIOA at day 18, were cultivated for 1 day with 30 ng M-CSF (**A**) and (**D**) (day 1) or with 50 ng for next 3 days (day 4) in (**B**) and (**E**) or with 50 ng M-CSF + 50 ng RANKL for next 3 days (day 7) in (**C**) and (**F**). One group was cultivated in the absence of AG490 and another in the presence of 50 mM AG490. Data are presented as means ± SD (n=7 per group in 3 experiments). **P < 0.01; *** P < 0.001, Student's unpaired t-test.*

was inhibited by tyrphostin. Such suppressive effect of AG490 was also noticed in regard to F4/80$^{high}$ cells in both, control and CIOA groups. Data showed that RANKL was expressed on cells from control (MFI=930±50) and CIOA mice (MFI=850±80) but the values of the MFI were lower in the presence of AG490 (MFI=640±60 and MFI=503±65 for control and CIOA groups, respectively) (Fig. 5B).

## DISCUSSION

In OA, changes in bone proceed together with cartilage deterioration, although it still remains unclear which of the two processes is responsible for the initial inflammatory disturbance. Progression of cartilage damage is commonly observed in human OA. OA is characterized by simultaneous presence of cartilage matrix degradation and regeneration/ repair, as well as by chondrocyte death, chondrocyte replication and proliferation.

However, animal models of OA show variations depending on induction methods and mouse strains. Recent findings point to an association between inflammation and disease appearance and progression. Anterior cruciate ligament transection and destabilization of the medial meniscus models are less related to strong inflammation in contrast to enzyme-induced instability models. In the CIOA

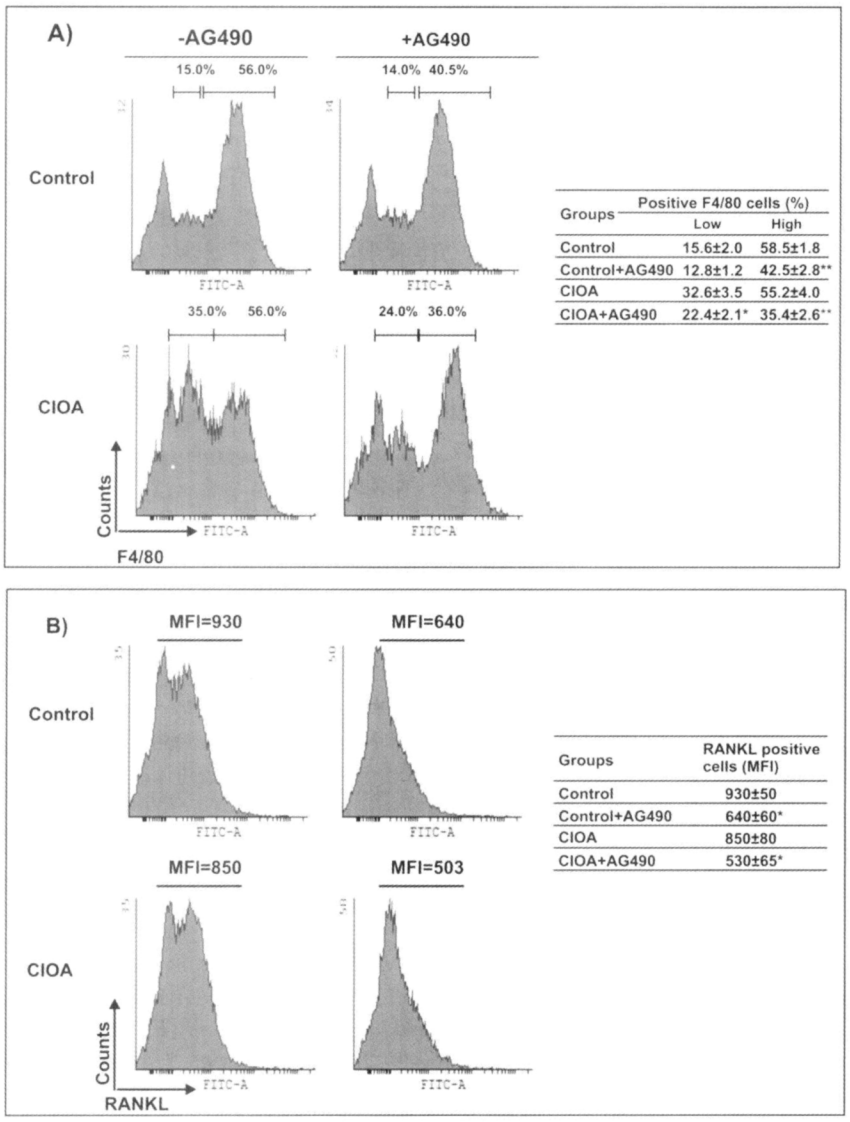



**Fig. 5.** *Effect of tyrphostin AG490 on M-CSF+RANKL-induced BM cell differentiation. BM cells were obtained from control and mice with CIOA at day 18, and stimulated with M-CSF+RANKL as described in Fig. 4.* ***A)*** *AG490 decreased the number of F4/80 positive cells on day 7. Data are presented as means ± SD (inserted table in A).* ***B)*** *AG490 lowered the expression of RANKL by BM cells at day 7. Data are presented as means ± SD (inserted table in B) (n=7 per group in 3 experiments). *P < 0.05; **P < 0.01, Student's unpaired t-test.*

model used in the present study, we observed cartilage impairment and elevated cytokine levels in the synovium at the early stage (days 3-7), and chronic inflammation, PG loss and increased bone turnover at day 30 (25). Of importance is the length of the follow-up period: in a longitudinal study an increased osteoclast activity and PG loss was observed 4 weeks after collagenase injection, but not 2 or 14 weeks after arthritis initiation (26).

We used a model of CIOA to evaluate the anti-osteoarthritic effect of tyrphostin AG490. The results showed marked decrease of cartilage destruction as a result of AG490 application. Bone erosion is based on the generation of osteoclasts within the synovial

membrane. This suggests that an intervention that protects bone from attack by inflammatory synovial tissue may be based on powerful inhibition of osteoclasts. At the late stage of CIOA we observed mature osteoclasts deep in the bone and their number was significantly decreased in AG490-treated mice. Proteoglycans and glycosaminoglycans are important articular cartilage matrix components considered sensitive to changes in cartilage homeostasis. GAG-rich extracellular matrices are reservoirs for growth factors that regulate cell development, adhesion, differentiation, and proliferation (27). Reduced density of toluidine blue and safranin O staining in CIOA mice demonstrated marked loss of GAGs and PGs in the cartilage, remarkably reduced in AG490-treated animals. The impairment of both cartilage architecture and tidemark zone were less exerted in mice injected with tyrphostin.

Locally produced cytokines are crucial for destroying articular tissue. Among these cytokines, TNF-α, IL-1β, and IFN-γ stimulate chondrocyte apoptosis and cartilage extracellular matrix degradation (28). The cytokines may play a critical role during both the induction and the established collagen-induced arthritis, first enhancing the immune response, and then regulating the arthritis process (29). AG490 inhibited IFN-γ levels in synovial fluid during onset of CIOA. This decrease caused by the substance might be associated with significantly reduced F4/80+ infiltration into joint tissue observed in tyrphostin-treated mice.

IL-1 and IL-6 are cytokines that play an important catabolic role in OA known to persist in high levels in the synovial fluid of patients with OA (30, 31). IL-1 is capable of suppressing chondrocyte proteoglycan synthesis in a model of zymosan-induced arthritis (32). In the same model, enhanced cartilage destruction was observed in IL-6-deficient mice together with reduced osteophyte formation (33). IL-6 could induce TGF-b which in turn resulted in marked osteophyte formation. In the early phase of arthritis IL-6 could reduce cartilage glycosaminoglycan loss, while in the chronic phase triggers osteophyte formation through induction of TGF-β3. We observed that AG490 attenuated the increase of IL-1 and IL-6 levels in the synovium during developing arthritis, while the late stage of CIOA was characterized with normal levels of IL-1. IL-6 is central also for the transition from the acute to the chronic phase of arthritis (34) which supposes that AG490 could alter such transition. Some investigations have also focused attention on the extent to which other pro-inflammatory cytokines, such as IL-17, activate JAK/STAT and the mechanism by which IL-17 modifies the production of IL-6 and other pro-inflammatory cytokines (35). AG490 significantly reduced IL-17 release in the SF during the whole period of CIOA development. IL-17 is capable of activating other signaling pathways, except JAK/STAT which resulted in elevated production of IL-1β and TNF-α. Therefore, the inhibition of IL-17 activity could also contribute to the reduction of these pro-inflammatory cytokines. Our data showed that CIOA is characterized by the presence of elevated number of neutrophils and macrophages in the synovial fluid (observed with F4/80 and CD11b/Ly6G flowcytometry staining), the cells underlying inflammation. As a result of tyrphostin treatment the number of these cell populations was close to that of the control mice. Except in the SF, we also established that the pool of PBMCs obtained in the chronic phase of CIOA consisted of elevated numbers of CD11b positive Ly6G cells, to a great extent reduced by AG490.

It is known that osteoclasts are enriched in the synovial membarane of patients with RA due to the presence of essential osteoclastogenic factors such as M-CSF and RANKL. They are formed by the fusion of hematopoietic cells of the monocyte-macrophage lineage and terminal differentiation is characterized by acquisition of mature phenotypic markers, such as expression of TRAP, matrix metallopeptidase-9 (MMP-9), and cathepsin K, leading to morphological conversion into large multinucleated cells and formation of resorption lacunae on bone (36). IL-6 is one of the major players in bone destructive events in various models of arthritis. In antigen-induced arthritis, IL-6 deficient mice developed less severe disease with fewer osteoclasts at sites of bone erosion compared with wild-type mice (37). IL-6 provoked osteoclast recruitment of hematopoietic stem cells from the granulocyte-macrophage lineage (38). In neonatal calvarial cultures, IL-6 induced enhanced expression of RANKL and OPG, but decreased RANK expression (39). Previously, we showed that tyrphostin AG490 suppressed M-CSF+RANKL-

induced osteoclast differentiation of BM cells *in vitro* (24). In the present experiments, we observed that the process of cell differentiation was attended with enhanced production of IL-1 and IL-6 by BM cells obtained from non-arthritic as well as from CIOA mice. The presence of AG490 during cultivation resulted in inhibition of this process. Thus, such environmental changes caused by tyrphostin AG490 might explain its anti-osteoclastogenic action. Moreover, AG490 down-regulated the expression of markers for F4/80 and RANKL on differentiated cells. This is consistent with the diminished presence of F4/80 positive cells in the synovial fluid and also with the previously established decrease of RANKL positive cells in the joints. It was observed that AG490 lowered the percentage of mature $F4/80^{high}$ population in both control and CIOA groups but lowered the percentage of maturing $F4/80^{low}$ cells only in the CIOA group.

In conclusion, tyrphostin AG490 ameliorates cartilage and bone destruction through decreased levels of critical inflammatory factors contributing to arthritis development such as IFN-γ, IL-1, IL-6 an IL-17 in the synovial fluid, and also can influence the processes of bone osteolysis. Previously, we established increased osteoclast activity associated with elevated RANKL expression in CIOA as well as in zymosan-induced arthritis (25). The latter is considered to be more relevant to rheumatoid arthritis than to OA. Even though these forms of arthritis are different, in their later stages, they come to resemble each other closely. Further investigations can answer the question whether tyrphostin AG490 will be effective in other models of OA or in RA-like conditions.

## ACKNOWLEDGEMENTS

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